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Systemic Antitumor Immunity in Experimental Brain Tumor Therapy Using a Multimutated, Replication-Competent Herpes Simplex Virus

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ABSTRACT

Replication-competent, attenuated herpes simplex virus (HSV) vectors have been developed for viral oncolytic therapy of primary and metastatic malignant brain tumors. However, the role of the host immune responses in the brain has not been elucidated. N18 neuroblastoma cells were used as a tumor model in syngeneic A/J mice to test the therapeutic efficacy of G207, a conditionally replicating HSV vector, in an immunocompetent condition. G207 inoculated intraneoplastically exhibited a prominent oncolytic antitumor effect in mice harboring N18 tumors in the brain or subcutaneously, and, in addition, elicited a systemic antitumor immune response. Subcutaneous tumor therapy with G207 caused regression of a remote, established tumor in the brain or in the periphery, which was potentially mediated by the systemic antitumor immune response, and provided persistent tumor-specific protection against N18 tumor rechallenge in the brain as well as in the periphery. Antitumor immunity was associated with an elevation of specific CTL activity against N18 tumor cells that persisted for at least 13 months. The results suggest that the oncolytic antitumor action of replication-competent HSV may be augmented by induction of specific and systemic antitumor immunity effective both in the periphery and in the brain.

OVERVIEW SUMMARY

G207 is a multimutated, conditionally replicating herpes simplex virus (HSV) vector developed for viral oncolytic therapy of malignant brain tumors. A/J mice harboring tumors of syngeneic N18 neuroblastoma were used to elucidate the role of the host immune responses in G207 tumor therapy. G207 inoculated intraneoplastically exhibited a prominent oncolytic antitumor effect against N18 tumors in the brain or skin. Subcutaneous tumor therapy with G207 elicited a systemic antitumor immune response that led to regression of a remote, established tumor in the brain or skin, and provided persistent tumor-specific protection against N18 tumor rechallenge in the brain and in the periphery. Antitumor immunity was associated with the elevation of specific and persistent CTL activity against N18 tumor cells. The oncolytic antitumor action of replication-competent HSV may be augmented by induction of specific and systemic antitumor immunity effective both in the periphery and in the brain.

INTRODUCTION

BRAIN TUMORS are the second leading cause of death from neurological disease, and the annual age-adjusted incidence of primary brain tumors in the United States has been reported to be 5.0 to 14.1 per 100,000 population (Radhakrishnan *et al.*, 1994). Gliomas account for 40–67% of the primary tumors, and about three-fourths of gliomas are considered malignant. Despite advances in microsurgical techniques and adjuvant therapy such as chemotherapy or radiotherapy, these modalities have caused little improvement in the survival rate of patients with malignant brain tumors. In addition, treatment of systemic tumors often fails owing to central nervous system (CNS) metastases. Genetically engineered, conditionally replicating viruses have been used to kill tumor cells by direct oncolysis (Martuza *et al.*, 1991; Markert *et al.*, 1993; Jia *et al.*, 1994; Kaplitt *et al.*, 1994; Mineta *et al.*, 1994, 1995; Chambers *et al.*, 1995; Kesari *et al.*, 1995; Bischoff *et al.*, 1996; Kramm *et al.*, 1997; Miyatake *et al.*, 1997; Pyles *et al.*, 1997; Rodriguez *et al.*, 1997). In this regard, herpes simplex virus (HSV) has ad-

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vantages for tumor therapy, including the ability to infect a variety of cell types, the availability of antiherpetic drugs to eradicate the virus, the identification of genes necessary for neuropathogenicity that can be mutated or deleted, and the large size of the HSV genomic DNA (150 kb) that allows for insertion of large or multiple genes. The first-generation attenuated HSVs had a mutation or a deletion in one gene. While these viruses proved to be efficient in killing tumor cells, neuropathogenicity was insufficiently attenuated, thereby raising concern about its use in humans (Markert *et al.*, 1993; Kesari *et al.*, 1995, 1998; Kramm *et al.*, 1996; Lasner *et al.*, 1996, 1998; McMenamin *et al.*, 1998).

G207, a second-generation, conditionally replicating HSV, has deletions in both copies of the *γ34.5* locus, a gene associated with neurovirulence (Chou *et al.*, 1990). G207 also has an insertion of the *Escherichia coli lacZ* gene in the *ICP6* gene (UL39), inactivating ribonucleotide reductase, an enzyme required for efficient viral DNA replication in nondividing cells but not in dividing cells (Goldstein and Weller, 1998). This double mutation confers favorable properties on G207 for treating human brain tumors: replication competence in tumor cells, markedly attenuated neurovirulence, ganciclovir/acyclovir hypersensitivity, and the presence of an easily detectable histochemical marker (β-galactosidase) (Mineta *et al.*, 1995). It has been shown that G207 kills various human tumor cells (glioma, malignant meningioma, breast cancer) in culture as well as in nude mice (Mineta *et al.*, 1995; Yazaki *et al.*, 1995; Toda *et al.*, 1998b). Yet, the effect of the immune system on the intracerebral antitumor action of G207, or any other genetically engineered HSV, has not been fully investigated. Only recently has the importance of the host immune response been recognized in viral oncolytic therapy for tumors outside the CNS (Toda *et al.*, 1998a, 1999).

Since the immune responses in the brain may differ from systemic responses, it is important to study the immune effects of HSV-induced tumor oncolysis in the brain. Therefore, we established an immunocompetent mouse brain tumor model with N18 murine neuroblastoma cells, which are moderately susceptible to G207 *in vitro* and are capable of forming both intracerebral and subcutaneous tumors in syngeneic A/J mice. G207 exhibits significant antitumor activity, potentially via induction of systemic antitumor immunity, in addition to the direct oncolytic activity by viral replication. Further, the specific antitumor immunity elicited by G207 treatment of a subcutaneous tumor not only provides protection against tumor rechallenge in the brain, but may also act to inhibit the growth or reduce the size of an established brain tumor.

MATERIALS AND METHODS

Viruses and cells

G207 was constructed as described (Mineta *et al.*, 1995) and contains deletions of both copies of the *γ34.5* gene as well as a *lacZ* insertion inactivating the *ICP6* gene. Stocks of G207 were grown in Vero (African green monkey kidney) cell cultures or provided by NeuroVir (Vancouver, Canada), and virus titers were determined as described previously (Miyatake *et al.*, 1997). The N18 cell line is a subclone of C1300 murine neuroblastoma cells derived from an A/J mouse ($H\text{-}2^a$) (Amano *et al.*, 1972), and was kindly provided by K. Ikeda (Tokyo Institute of Psychiatry, Tokyo, Japan). SR-B10.A and 203GL murine glioma cell lines were obtained from the Department of Neurosurgery, University of Tokyo (Tokyo, Japan). SCK mammary carcinoma cells, derived from a tumor that spontaneously arose in an A/J mouse (Song *et al.*, 1980), were kindly provided by W.M.F. Lee (University of Pennsylvania, Philadelphia, PA). These cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 m*M* glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (Fungizone, 2.5 μg/ml). SaI/N, a chemically induced sarcoma cell line from an A/J mouse (Jutila *et al.*, 1962), was kindly provided by S. Ostrand-Rosenberg (University of Maryland Baltimore County, Baltimore, MD), and maintained in minimum essential medium supplemented as described above.

In vitro *cytotoxicity studies*

Cells were plated in six-well plates at 2×10^5 cells/well. After a 24-hr incubation at 37°C, cells were infected with G207 at various multiplicities of infection (MOIs of 0.01 to 1) or virus buffer (150 m*M* NaCl, 20 m*M* Tris, pH 7.5), and further incubated at 34.5°C. The number of surviving cells was counted daily with a Coulter counter (Beckman Coulter, Fullerton, CA). Before counting, cells were washed twice with 1 ml of phosphate-buffered saline (PBS) to eliminate floating cells, and then trypsinized.

Animal studies

Six-week-, 3-month-, or 10-month-old female A/J mice were purchased from the National Cancer Institute (Frederick, MD) and caged in groups of four or less. For injections and surgical procedures, each mouse was anesthetized with an intraperitoneal injection of 0.20–0.25 ml of solution consisting of 86% saline, 9% sodium pentobarbital, and 5% ethyl alcohol. All animal procedures were approved by the Georgetown University Animal Care and Use Committee.

Subcutaneous tumor therapy

Subcutaneous tumors were generated by injecting 5×10^6 N18 cells in 50 μl of serum-free medium subcutaneously into the left or bilateral flank(s) of 6-week-old female A/J mice. When subcutaneous tumors reached approximately 6 mm in diameter, 20 μl of G207 at various concentrations or mock-infected extract was inoculated intraneoplastically into the left tumor. Mock-infected extract was prepared from virus buffer-infected cells, using the same procedures as those used for virus inoculum. In some experiments, the treatment was repeated on day 3. Tumor growth was determined by measuring the tumor volume (length × width × height) twice a week.

Intracerebral tumor therapy

Intracerebral tumors were generated by injecting 5×10^5 N18 cells in 5 μl of serum-free medium stereotactically into the right frontal lobe of A/J mice. After 7 days, 5 μl of G207 (1.5×10^7 plaque-forming units [PFU]) or mock-infected extract was inoculated stereotactically at the same coordinates, and survival was monitored.

Rechallenge studies

Three groups of A/J mice were used for the studies: (1) the naive group; (2) the spontaneous rejection group, mice that were subcutaneously injected with N18 cells but did not develop a tumor or spontaneously rejected a tumor without any treatment; and (3) the G207-cured group, mice with established subcutaneous N18 tumors that had been cured by intraneoplastic G207 inoculation.

For subcutaneous rechallenge studies, 5×10^6 N18 cells or 5×10^5 SaI/N cells in 50 μl of serum-free medium were injected subcutaneously into the right flank region, and tumor growth was determined as described above.

For intracerebral rechallenge studies, 5×10^5 N18 cells in 5 μl of serum-free medium were stereotactically injected into the right frontal lobe, and survival was monitored.

Subcutaneous tumor treatment in a simultaneous intracerebral and subcutaneous tumor model

N18 cells (5×10^6 cells in 50 μl of serum-free medium) were injected subcutaneously into A/J mice in the left flank region on day 0. On day 3, N18 cells (5×10^5 cells in 5 μl of serum-free medium) were injected stereotactically into the right frontal lobe. On day 10, subcutaneous tumors were treated with intraneoplastic inoculations of either G207 (1×10^7 PFU) or mock-infected extract (n = 11/group). The treatment was repeated on day 13, and survival was monitored. Animals were sacrificed by intraperitoneal injections of sodium pentobarbital when moribund from the enlarged intracerebral tumor, when the size of the subcutaneous tumor exceeded 24 mm in maximal diameter, or when the subcutaneous tumor developed ulceration.

Detection of G207 by polymerase chain reaction analysis

Of the mice in the simultaneous intracerebral/subcutaneous tumor model group, whose subcutaneous tumors were treated with G207, one that died of a brain tumor (mouse 1) and another that was sacrificed owing to a large subcutaneous tumor (mouse 2, brain tumor cured) were analyzed by polymerase chain reaction (PCR) for the presence of G207 DNA in the subcutaneous tumor or the brain. DNA was extracted from the right frontal lobe of the brain (divided into two pieces) and from four specimens from the subcutaneous tumor, using a QIAamp tissue kit (Qiagen, Santa Clarita, CA) and following the manufacturer protocol. PCR was performed in 100-μl volumes (containing 10 μl of extracted DNA sample, 2 mM $MgCl_2$, a 200 μM concentration of each deoxynucleoside triphosphate, PCR buffer II [Perkin-Elmer, Branchburg, NJ], a 1 μM concentration of each primer, and 2.5 U of *Taq* polymerase) for 35 cycles (denaturation at 94°C for 90 sec, annealing at 55°C for 60 sec, and extension at 72°C for 120 sec), using a PTC-100 thermal controller (MJ Research, Watertown, MA). A primer pair sequence was selected to amplify the *lacZ* portion of the G207 DNA with an expected product size of 300 base pairs (bp) (Ramakrishnan *et al.*, 1994). A primer pair to amplify fatty acid-binding protein sequences with an expected product size of 127 bp was also used as a control (Kurtz *et al.*, 1994).

Detection of G207 by X-Gal histochemistry

Tumor-containing brain and subcutaneous tumor were harvested from two mice of the control group and two mice of the G207-treated group in the simultaneous intracerebral/subcutaneous tumor experiment 5 days after the first G207 inoculation into the subcutaneous tumor, and also from the same number of animals from each group 10 days postinoculation. The samples were snap frozen in isopentane cooled with dry ice. Cryostat sections, 10 μm in thickness, were prepared from each sample. Sections were fixed in 2% paraformaldehyde in PBS for 10 min, washed three times in PBS, and incubated with PBS containing 2 mM magnesium chloride, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 (NP-40) at 4°C for 10 min. Sections were further incubated with substrate solution (PBS containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal, 1 mg/ml], 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% sodium deoxycholate, and 0.02% NP-40) at 32°C for 3 hr, and then washed once with water and twice with PBS containing 2 mM EDTA. Sections were counterstained with hematoxylin and eosin before mounting.

CTL assays

A/J mice with established subcutaneous N18 tumors of approximately 6 mm in diameter in the left flank region were treated with intraneoplastic inoculations of 20 μl of G207 (1×10^7 PFU) or mock-infected extract, and the treatment was repeated 3 days later. Fourteen days after the first treatment, three mice from each treatment group and from the "spontaneous rejection group" were sacrificed and the spleens harvested. In one experiment, spleens from three mice 13 months after subcutaneous tumor therapy (cured by G207 treatment) were also harvested.

In 24-well plates, red blood cell-depleted spleen cell suspensions (3×10^6 cells) were cocultured with 1×10^6 γ-irradiated (50 Gy) N18 cells/well in 2 ml of complete medium (RPMI 1640 supplemented with 10% FCS, penicillin [100 U/ml], streptomycin [100 μg/ml], Fungizone [2.5 μg/ml], 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μM 2-mercaptoethanol) for 5 days at 37°C. After this incubation period, spleen cells were harvested, dead cells were removed by centrifugation over Fico/Lite-LM (Atlanta Biologicals, Norcross, GA), and viable cells were collected and washed three times with medium. Target cells (N18 or SCK; 10^6) were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, IL) for 60 min and washed three times with medium. Effector spleen cells were added to wells of a 96-well U-bottomed microtiter plate containing 10^4 labeled target cells in complete medium at the effector-to-target ratios indicated. To determine spontaneous and total release of radioactivity, the same number of target cells was incubated with culture medium alone or 0.1 M HCl. After a 4-hr incubation at 37°C, the supernatant from each well was recovered with a Skatron (Lier, Norway) Titertek system and counted in a γ counter for ^{51}Cr release. The percentage of specific lysis was calculated from triplicate samples as follows: percent specific lysis = [(experiment cpm − spontaneous cpm)/(maximum releasable cpm − spontaneous cpm)] × 100.

Flow cytometric analysis

Single-cell suspensions of splenocytes (1×10^6 cells) were washed once with 2 ml of PBS containing 2% FCS and 0.1% sodium azide, and incubated with 100 μl of fluorescein isothiocyanate (FITC)- or R-phycoerythrin (PE)-conjugated monoclonal antibody diluted in PBS at a saturating concentration for 30 min at 4°C. The monoclonal antibodies used were FITC-conjugated anti-mouse CD3 (clone 145-2C11), PE-conjugated anti-mouse CD4 (clone GK1.5), PE-conjugated anti-mouse CD8 (clone 53-6.7), PE-conjugated anti-mouse CD19 (clone 1D3), and PE-conjugated anti-mouse natural killer (NK) cells (clone DX5), all purchased from PharMingen (San Diego, CA). After two washes with PBS, cells were fixed in 1 ml of PBS containing 0.5% paraformaldehyde, and analyzed on a FACScan flow cytometer, using the lymphocyte gate (Becton Dickinson, Mountain View, CA).

RESULTS

Cytopathic effect of G207 on murine tumor cells in vitro

Murine tumors do not support the replication of HSV vectors as well as human tumors (Lopez, 1975; Zawatzky *et al.*, 1981). Thus, most prior studies of HSV tumor therapy have been performed in human tumors in immunocompromised mice (Chambers *et al.*, 1995; Kesari *et al.*, 1995; Mineta *et al.*, 1995; Yazaki *et al.*, 1995; Lasner *et al.*, 1996; Pyles *et al.*, 1997; Toda *et al.*, 1998b). Therefore, to establish a brain tumor model suitable for evaluating the antitumor efficacy of G207 as well as other replication-competent HSV vectors in an immunocompetent syngeneic mouse, we investigated the susceptibility of various nervous system-related murine tumor cell lines to G207. Among the cell lines studied, N18, a subclone of C1300 murine neuroblastoma cells that arose spontaneously from an A/J mouse (Amano *et al.*, 1972; Ziegler *et al.*, 1997), proved to be the most susceptible to G207 replication and spread (Fig. 1). G207 killed more than 90% of the N18 cells when infected at an MOI of 1 (3 days postinfection) and about 40% of the cells at an MOI of 0.1 (4 days postinfection) (Fig. 1, left). N18 cells are moderately susceptible to G207, although less sensitive than human cell lines reported previously (Mineta *et al.*, 1995; Yazaki *et al.*, 1995; Toda *et al.*, 1998b). In comparison, SR-B10.A, a B10.A mouse-derived glioma cell line, was only mildly susceptible to G207, exhibiting 75 and 24% cell death at MOIs of 1 and 0.1, respectively, 3 days postinfection (Fig. 1, middle), and 203GL glioma cells were only minimally susceptible (Fig. 1, right).

G207 treatment of subcutaneous N18 tumors in A/J mice

Results from subcutaneous injections of various number of N18 cells (1×10^5 to 1×10^7 cells) into the flank region of 6-week-old female A/J mice indicated that reproducible subcutaneous tumor formation is achieved with 5×10^6 cells, with a take rate of approximately 90% (data not shown). A growing tumor approximately 6 mm in diameter is evident within 7 to 10 days of tumor cell implantation, and ulcer formation is rarely encountered during tumor growth.

To test the antitumor efficiency of G207 in an immunocompetent host, 7-week-old A/J mice harboring established subcutaneous N18 tumors (approximately 6 mm in diameter) were treated with either G207 (1.5×10^7 pfu) or mock-infected extract ($n = 6$ per group). A single intraneoplastic inoculation of G207 efficiently inhibited tumor growth or reduced tumor

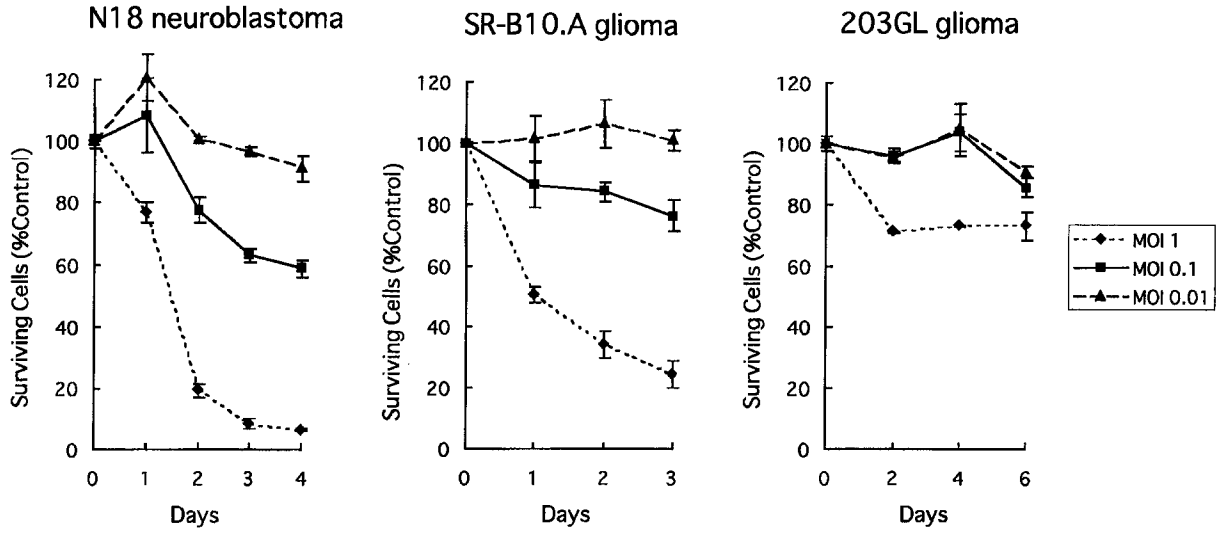


FIG. 1. Cytopathic effect of G207 on nervous system-related murine tumor cells *in vitro*. Cells (N18, SR-B10.A, and 203GL) were plated into six-well plates at 2×10^5 cells/well. After a 24 hr-incubation, cells were infected with G207 at various multiplicities of infection (MOI, 0.01–1) or virus buffer (mock). The number of surviving cells was counted daily and expressed as a percentage of mock-infected controls. N18 cells showed a moderate susceptibility to G207, whereas the other two cell lines showed a mild to minimal susceptibility. The results represent the mean of triplicates ± SD.

size (Fig. 2). On day 18 posttreatment, when the experiment was terminated owing to tumor burden in control animals, the G207-treated mice had a significantly smaller mean tumor volume compared with mock-treated mice ($p < 0.05$, unpaired t test). Three of six G207-treated mice were cured, whereas all six mock-treated mice showed continuous tumor growth. The cured mice remained tumor free for 9 months until the observation was terminated.

To investigate whether the effect is dose dependent, established subcutaneous N18 tumors were treated with intraneoplastic inoculations of G207 on days 0 and 3 at doses ranging from 1×10^3 to 1×10^5 PFU or mock-infected extract ($n = 7$ per group). Inoculations of G207 resulted in a dose-dependent inhibition of tumor growth *in vivo* (Fig. 2B). G207 at 1×10^3 PFU did not cause a significant inhibition of tumor growth compared with mock-treated controls. G207 at 1×10^4 PFU resulted in a smaller mean tumor volume than in controls on day 21, although the difference was not significant ($p = 0.07$, unpaired t test), and 1×10^5 PFU of G207 caused a significant decrease in tumor size compared with mock-treated controls ($p < 0.05$ on day 21, unpaired t test).

G207 treatment of intracerebral N18 tumors in A/J mice

To investigate the antitumor efficacy of G207 in the brain in an immunocompetent mouse model, 5×10^5 N18 cells were injected stereotactically into the right frontal lobe of 6-week-old A/J mice. These cells generate a reproducible brain tumor model in which an intraparenchymal tumor is formed that causes death in 2 to 3 weeks. Seven days after the tumor implantation, G207 (1.5×10^7 PFU) or mock-infected extract was inoculated intraneoplastically ($n = 15$ and 16, respectively), and survival was observed. A single inoculation of G207 caused a significant prolongation of survival compared with mock-inoculated controls, with three long-term survivors or "cures" ($p = 0.018$, Wilcoxon test, Fig. 3). Necropsies showed that all animals that died within 60 days of tumor implantation had enlarged brain tumors. Histopathology of the brain at the time of death typically showed a large intraparenchymal tumor mass consisting of small, round, cytoplasm-scarce N18 neuroblastoma cells occupying a large proportion of the right hemisphere with a severe midline shift to the left (example shown in Color Plate 1A). In contrast, histopathology of serial sections of the brain from a G207-treated, long-term survivor 18 months after treatment revealed gliosis and hyaline change, but no residual tumor cells, at the site of tumor cell implantation (Color Plate 1B).

Intraneoplastic inoculations of G207 elicit systemic antitumor immunity

A/J mice harboring established bilateral subcutaneous N18 tumors were used to determine the role of the immune response in the antitumor activity of G207. Six-week-old A/J mice were injected with 5×10^6 N18 cells subcutaneously into bilateral flanks. When both subcutaneous tumors reached approximately 6 mm in diameter, the left tumor alone was treated with an intraneoplastic inoculation of G207 (1×10^7 PFU) or mock-infected extract ($n = 8$ per group), followed by a second inocu-

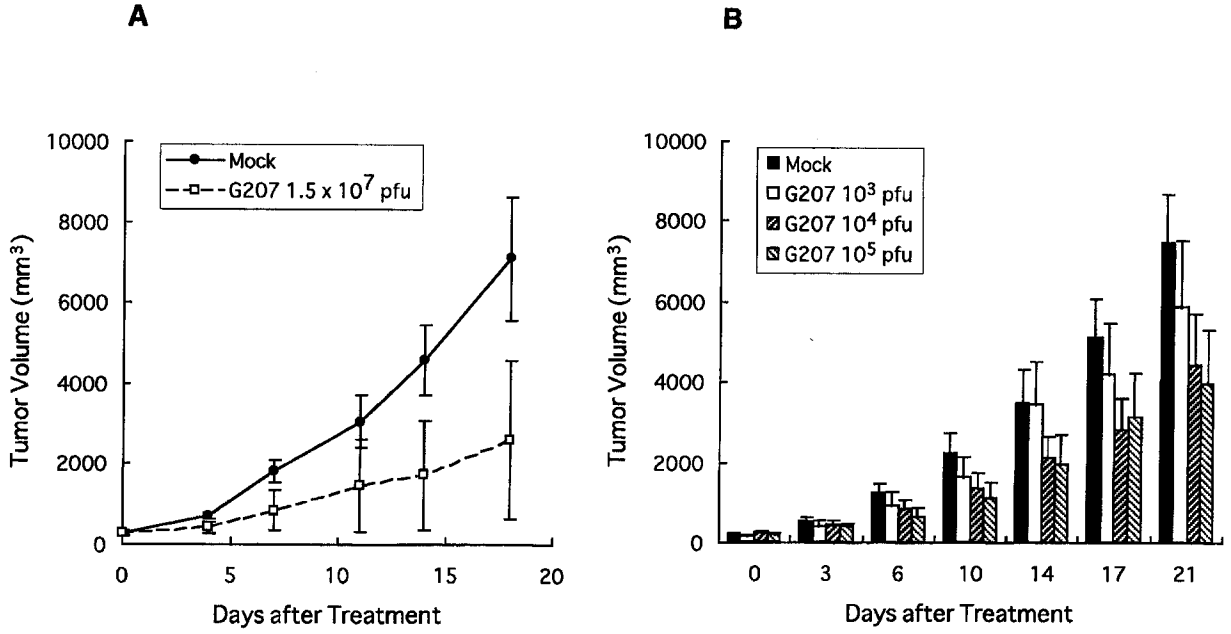


FIG. 2. Intraneoplastic inoculation of G207 causes a reduction in tumor growth in A/J mice harboring subcutaneous N18 tumors. Seven-week-old A/J mice harboring subcutaneous N18 tumors of approximately 6 mm in diameter were treated with either G207 or mock-infected extract. **(A)** A single intraneoplastic inoculation of G207 (1.5×10^7 PFU) significantly inhibited tumor growth compared with the mock-treated control ($n = 6$ per group, $p < 0.05$ on day 18, unpaired t test). **(B)** Subcutaneous tumors were inoculated with G207 at lower doses ranging from 1×10^3 to 1×10^5 PFU or with mock-infected extract, which was repeated on day 3 ($n = 7$ per group). Inoculations of G207 resulted in a dose-dependent inhibition of the tumor growth. The results represent the mean ± SEM. Tumor volume = length × width × height.

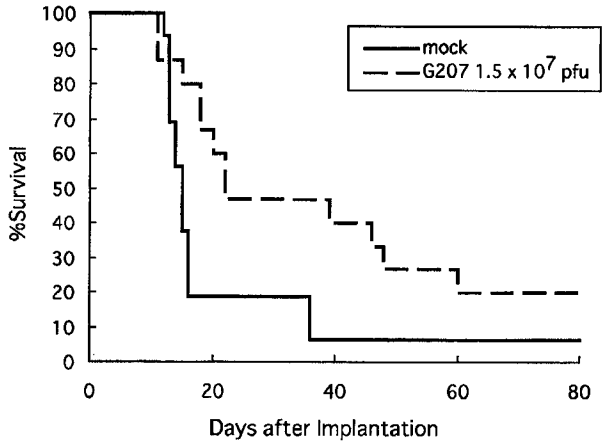


FIG. 3. Intraneoplastic inoculation of G207 prolongs the survival of A/J mice bearing intracerebral N18 tumors. N18 cells (5×10^5) were injected stereotactically into the right frontal lobe of 6-week-old A/J mice. Seven days after tumor implantation, G207 (1.5×10^7 PFU) or mock-infected extract was inoculated stereotactically at the same coordinates ($n = 15$ and 16, respectively). A single inoculation of G207 caused a significant prolongation of survival compared with mock-inoculated controls ($p = 0.018$ by Wilcoxon text).

lation 3 days later. G207 inoculations caused a significant reduction in tumor growth, not only of the inoculated tumors, but also of tumors on the contralateral, noninoculated side ($p < 0.05$ on day 23 for both sides compared with mock, unpaired t test; Fig. 4). Both bilateral (inoculated and noninoculated) tumors in G207-inoculated animals exhibited similar inhibition of tumor growth, and six of eight G207-treated mice showed disappearance of both tumors within 23 days and remained tumor free for 5 months until the observation was terminated. All eight mock-treated mice exhibited continuous growth of the bilateral tumors. As we have demonstrated in other models (Toda *et al.*, 1999), this result suggests that an intraneoplastic inoculation of G207 into a subcutaneous tumor on one side caused a systemic antitumor immune response that resulted in a reduction of the growth of a remote, noninoculated tumor on the contralateral side.

To test whether an intraneoplastic inoculation of G207 confers protective antitumor immunity with memory, 12 A/J mice (ages 4 to 9 months) that once bore subcutaneous N18 tumors that were cured by intraneoplastic G207 inoculations were rechallenged with a subcutaneous injection of 5×10^6 N18 cells (G207-cured group). For comparison, 30 A/J mice of the same age range that had spontaneously rejected their tumors without treatment were also rechallenged with N18 cells (spontaneous rejection group). Ten 3-month-old naive A/J mice were used as

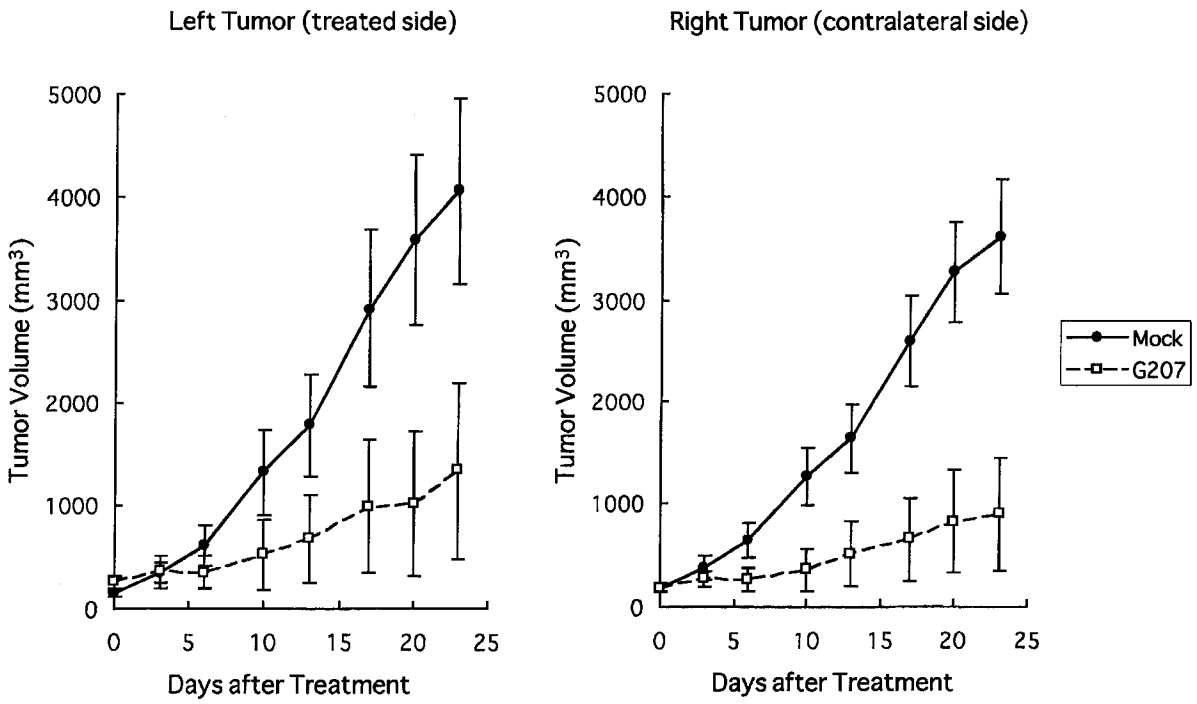
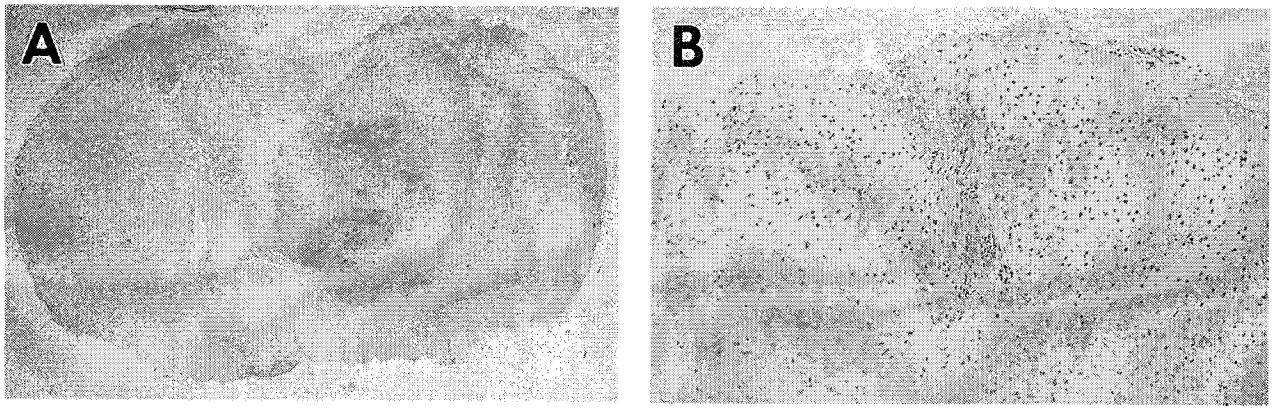


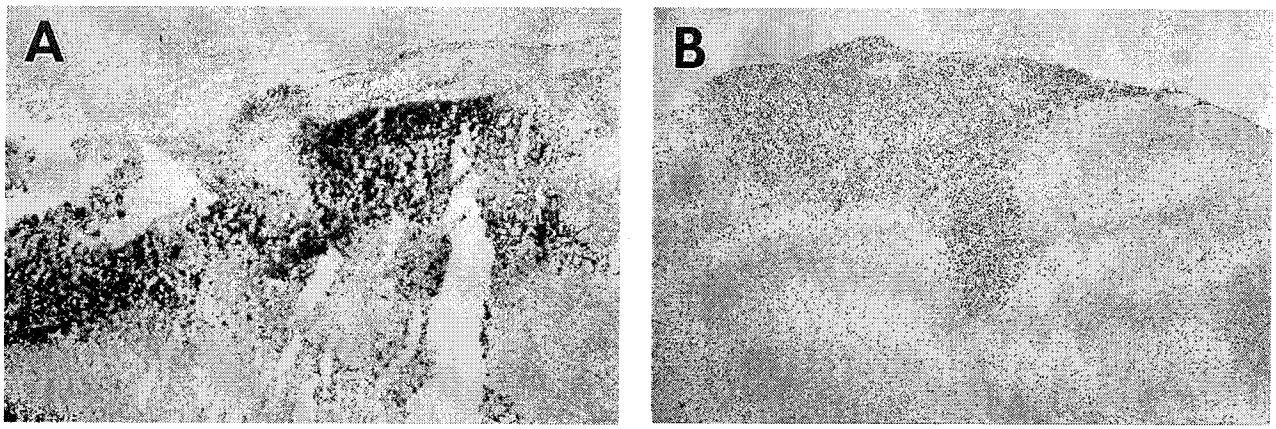
FIG. 4. G207 inoculations cause regression of inoculated subcutaneous tumors as well as noninoculated contralateral tumors. N18 cells (5×10^6) were injected subcutaneously into bilateral flanks of 6-week-old A/J mice. When both subcutaneous tumors reached approximately 6 mm in diameter, the left tumor alone was treated with an intraneoplastic inoculation of G207 (1×10^7 PFU) or mock-infected extract ($n = 8$ per group). The treatment was repeated on day 3. G207 inoculations caused a significant reduction in tumor growth of inoculated tumors (left) and also of noninoculated tumors on the contralateral side (right) compared with respective controls ($p < 0.05$ on day 23 for both sides, unpaired t test). The results represent the mean ± SEM. Tumor volume = length × width × height.



COLOR PLATE 1. Histopathology of intracerebral N18 tumor. **(A)** Moribund A/J mouse sacrificed 16 days after a stereotactic injection with 5×10^5 N18 cells into the right frontal lobe. The brain was fixed in 10% formalin, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin. A large intraparenchymal tumor mass consisting of small, round, cytoplasm-scarce N18 neuroblastoma cells occupying a large proportion of the right hemisphere with a severe midline shift to the left and a compression of ventricles is shown. **(B)** Serial sections of the brain from a G207-treated, long-term survivor 18 months after the treatment showed gliosis and hyaline change at the site of tumor cell injection. [H&E; original magnification: **(A)** $\times 8$; **(B)** $\times 75$.]

controls (naive group). The tumor growth in individual animals after rechallenge with a subcutaneous injection of N18 cells is shown in Fig. 5. In the naive group, five mice showed continuous tumor growth and, in the other five, the tumors temporarily grew to a tumor size larger than 75 mm^3 and then regressed. In the spontaneous rejection group, 4 of 30 mice had continuous tumor growth, 14 had temporary growth (>75 mm^3), and 12 had no tumor growth. In contrast, all 12 mice of the G207-cured group showed no sign of tumor growth throughout the observation period of 6 months, indicating the presence of strong and persistent protective antitumor immunity.

To prove that this systemic antitumor immunity elicited by G207 treatment was specific for N18 tumor cells, the survivors from the subcutaneous N18 rechallenge studies described above were further challenged with a subcutaneous injection of 5×10^5 SaI/N, A/J-derived sarcoma cells. Fourteen 10-month-old naive A/J mice were used as controls. By 6 weeks after SaI/N cell implantation, subcutaneous tumor formation was observed in 7 of 14 mice in the 10-month-old naive group, 6 of 12 mice in the G207-cured group, and 10 of 21 mice in the spontaneous rejection group, showing no significant difference between the three groups (χ^2 test). The fact that the antitumor immunity induced by G207 against N18 cells did not affect the growth of SaI/N cells indicates that the antitumor immune response was tumor specific. A decrease in take rate of SaI/N subcutaneous tumors in aged A/J mice that we observed in this and other stud-



COLOR PLATE 2. X-Gal histochemistry of G207-inoculated subcutaneous N18 tumor and coexisting, noninoculated N18 brain tumor. In mice bearing established subcutaneous and intracerebral N18 tumors simultaneously, the subcutaneous tumor only was treated with an intraneoplastic inoculation of G207 (1×10^7 PFU), and repeated 3 days later. Five and 10 days after the first inoculation, the subcutaneous tumor and the brain were harvested and stained with X-Gal to detect G207. **(A)** An abundant expression of β-galactosidase (*lacZ*) is observed in the G207-inoculated subcutaneous tumor harvested 5 days postinoculation. **(B)** No histochemical staining is detected in the remote, noninoculated brain tumor from the same animal. [Counterstained with H&E. Original magnification: **(A** and **B)** $\times 30$.]

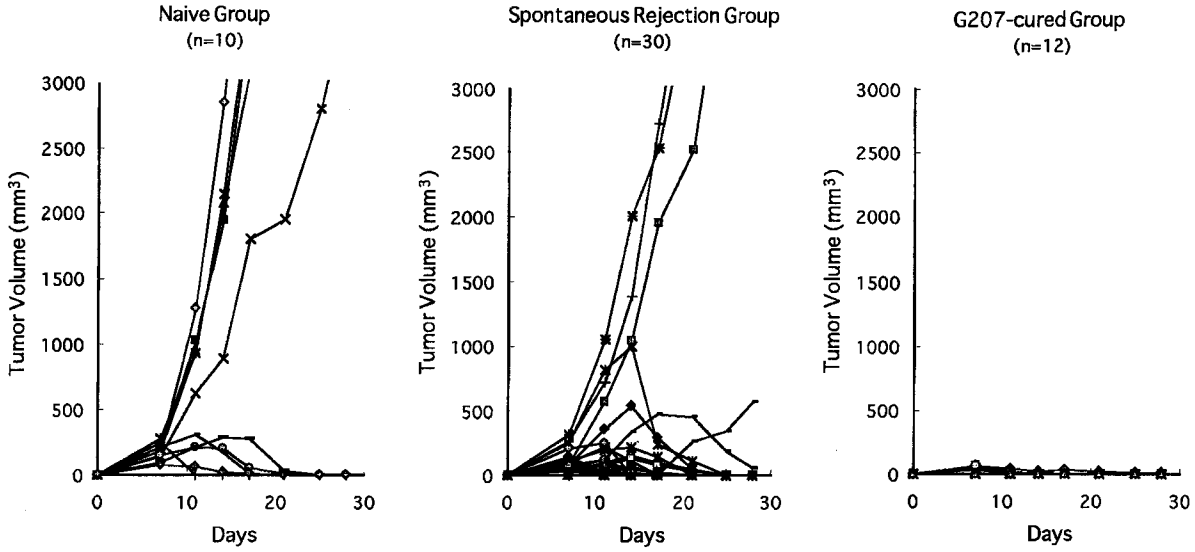


FIG. 5. G207 treatment of subcutaneous tumors confers protection against subcutaneous tumor rechallenge. Naive A/J mice (naive group, $n = 10$), A/J mice that spontaneously rejected N18 tumor cell implants without treatment (spontaneous rejection group, $n = 30$), and A/J mice whose established subcutaneous N18 tumors had been cured by intraneoplastic G207 inoculations (G207-cured group, $n = 12$) were rechallenged with subcutaneous injections of 5×10^6 N18 cells into the right flank region. The tumor volume of each mouse is shown individually. None of the G207-cured group showed any sign of tumor growth, while five of the naive group and four of the spontaneous rejection group showed continuous tumor growth. Tumor volume = length × width × height.

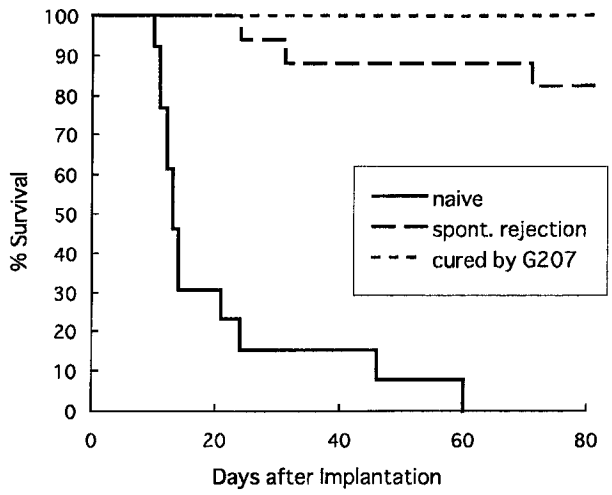


FIG. 6. G207 treatment of subcutaneous tumors confers protection against intracerebral tumor rechallenge. Naive A/J mice (naive group, $n = 13$; —), A/J mice that spontaneously rejected N18 tumor cell implants without treatment (spontaneous rejection group, $n = 17$; – – –), and A/J mice whose established subcutaneous N18 tumors had been cured by intraneoplastic G207 inoculations (G207-cured group, $n = 11$; - - -) were rechallenged with intracerebral injections of 5×10^5 N18 cells into the right frontal lobe. All 13 mice of the naive group died within 60 days and 3 of 17 mice of the spontaneous group died within 80 days. In contrast, all of the G207-cured group survived for more than 12 months ($p < 0.001$ versus naive group and $p = 0.15$ versus spontaneous rejection group, Wilcoxon test).

ies (data not shown) is in keeping with descriptions by others that old animals are less susceptible than young animals as hosts for tumor xenografts (Fodstad, 1991).

To test whether the systemic antitumor immunity elicited by intraneoplastic G207 inoculation into subcutaneous tumors also provided protective immunity to a tumor within the brain, an organ considered by some to have an immune-privileged environment, adult A/J mice (3–5 months old) with or without previous G207 treatment were rechallenged with an intracerebral injection of 5×10^5 N18 cells. The previous subcutaneous tumor therapy was performed when animals were 6 weeks old. All 13 mice of the naive group died within 60 days and 3 of 17 mice of the spontaneous rejection group died within 80 days of rechallenge (82% survival) (Fig. 6). Necropsies showed that all of these animals died from brain tumor development. In contrast, none of 11 mice of the G207-cured group died from a developing brain tumor and all survived for more than 12 months after rechallenge. The antitumor immunity elicited by intraneoplastic G207 inoculation, therefore, was shown to provide efficient and persistent protective antitumor immunity in the brain as well.

Intraneoplastic inoculations of G207 induce specific and persistent antitumor CTL activity

We next investigated whether the systemic antitumor immunity elicited by an intraneoplastic inoculation of G207 is associated with an induced CTL activity specific for N18 tumor cells. Seven-week-old A/J mice harboring subcutaneous N18 tumors of approximately 6 mm in diameter were treated with

TABLE 1. EFFECT OF INTRANEOPLASTIC G207 INOCULATIONS ON SPLENOCYTE SUBSETS[a]

	Before restimulation[b]			*After restimulation*[b]		
Phenotype	*Mock treated*	*G207 treated*	*Spontaneous rejection*	*Mock treated*	*G207 treated*	*Spontaneous rejection*
$CD3^+$ cells	22.6	33.8	30.4	46.8	75.5	66.0
$CD4^+$ cells	12.7	18.7	15.5	38.7	54.0	41.5
$CD8^+$ cells	8.0	12.7	12.8	7.4	22.5	26.7
$CD19^+$ cells	64.8	57.8	62.7	49.5	22.6	32.4
NK cells	1.5	1.9	1.5	1.1	0.4	0.4

[a]A/J mice with established subcutaneous N18 tumors of approximately 6 mm in diameter were treated with intraneoplastic inoculations of G207 (1×10^7 PFU) or mock-infected extract on days 0 and 3. Fourteen days after the first treatment, the spleens were harvested from three mice from each treatment group and from the spontaneous rejection group, defined as mice that were subcutaneously injected with N18 cells but did not develop a tumor. The splenocytes were cocultured with irradiated N18 cells for 5 days for restimulation. Splenocytes of the three mice from the same treatment group were pooled, and flow cytometric analyses performed before and after the restimulation.

[b]Values listed represent the percentage of marker-positive cells.

an intraneoplastic inoculation of either mock-infected extract or G207 (1×10^7 PFU). The treatment was repeated on day 3. Fourteen days after the first treatment, three mice from each group were sacrificed and spleens were harvested (mock-treated and G207-treated groups). For comparison, three mice that received a subcutaneous injection of N18 cells but did not develop an established tumor were also included in the studies (spontaneous rejection group). Splenocytes from individual animals were cocultured separately with irradiated (50 Gy) N18 cells for 5 days for restimulation.

Flow cytometric analyses were performed on splenocytes, pooled within the same group, before and after the coculture to verify the cell subset profile. A large increase in the proportion of CD3-positive T cells and a decrease in CD19-positive B cells were noted in the G207-treated group compared with the mock-treated group (Table 1). An increase in both CD4- and CD8-positive cells accounted for the increase in CD3-positive T cells. These differences were enhanced after the 5-day coculture with irradiated N18 cells. The splenocyte subset profile of the spontaneous rejection group lay in between those of the mock-treated group and the G207-treated group.

The effector cells generated after coculture were applied to labeled N18 target cells in a ^{51}Cr release assay. Effector cells harvested from individual animals were assayed separately (n = 3 per group). The effector cells of the G207-treated group exhibited a significantly increased, dose-dependent CTL activity against the target N18 cells compared with those of the mock-treated or spontaneous rejection groups ($p < 0.05$ at an effector-to-target [*E/T*] ratio of 100:1, unpaired t test; Fig. 7A). The spontaneous rejection group showed an intermediately elevated CTL activity against N18 cells, although not significant compared with the mock-treated group ($p = 0.15$ at an *E/T* ratio of 100:1). Similar results were obtained from two other experiments (data not shown), confirming a prominent induction of N18-targeted CTL activity by intraneoplastic inoculations of G207.

To demonstrate the specificity of the induced CTL activity, effector cells were applied to labeled SCK, A/J-derived mammary carcinoma target cells. The effector cells from all three groups showed a dose-dependent lysis of SCK target cells to some extent, but, unlike the results with N18 target cells, no difference in the level of lysis was observed between the three groups at any *E/T* ratio (Fig. 7B). Hence, the highly elevated CTL activity against N18 cells shown by the effector cells of the G207-treated group was specific for N18 tumor cells.

To investigate further whether long-term memory exists for the N18 tumor-specific CTL activity, three 15-month-old mice whose established subcutaneous N18 tumors had been cured by intraneoplastic G207 inoculations 13 months earlier were examined. A ^{51}Cr release assay revealed the presence of a significantly increased, dose-dependent CTL activity against N18 target cells with these aged mice (Fig. 7A). Even 13 months after subcutaneous tumor therapy with G207, the CTL activity level was identical to that of the 2-month-old mice 14 days after G207 treatment. These results indicate that once specific antitumor immunity is established by intraneoplastic G207 inoculations, immunologic memory persists for a long time even in aged mice.

Treatment of established N18 brain tumors via induction of specific antitumor immunity

To investigate whether an established brain tumor can be treated via the systemic antitumor immunity induced by an intraneoplastic inoculation of G207 into a remote subcutaneous tumor, mice bearing established subcutaneous and intracerebral tumors simultaneously were utilized. The model was generated by a subcutaneous injection of 5×10^6 N18 cells into 6-week-old A/J mice in the left flank region (day 0), followed by a stereotactic intracerebral injection of 5×10^5 N18 cells into the right frontal lobe on day 3. On day 10, subcutaneous tumors were treated with an intraneoplastic inoculation of either mock-infected extract or G207 (1×10^7 PFU) (n = 11 per each group), and repeated on day 13. Intraneoplastic inoculations of G207 into a subcutaneous tumor caused growth suppression of the inoculated subcutaneous tumor and also of the remote intracerebral tumor, leading to a significant prolongation of survival compared with mock-inoculated control animals (p = 0.032, Wilcoxon test; Fig. 8). All 11 mock-treated mice either died from or showed intracerebral tumor growth, whereas 4 of

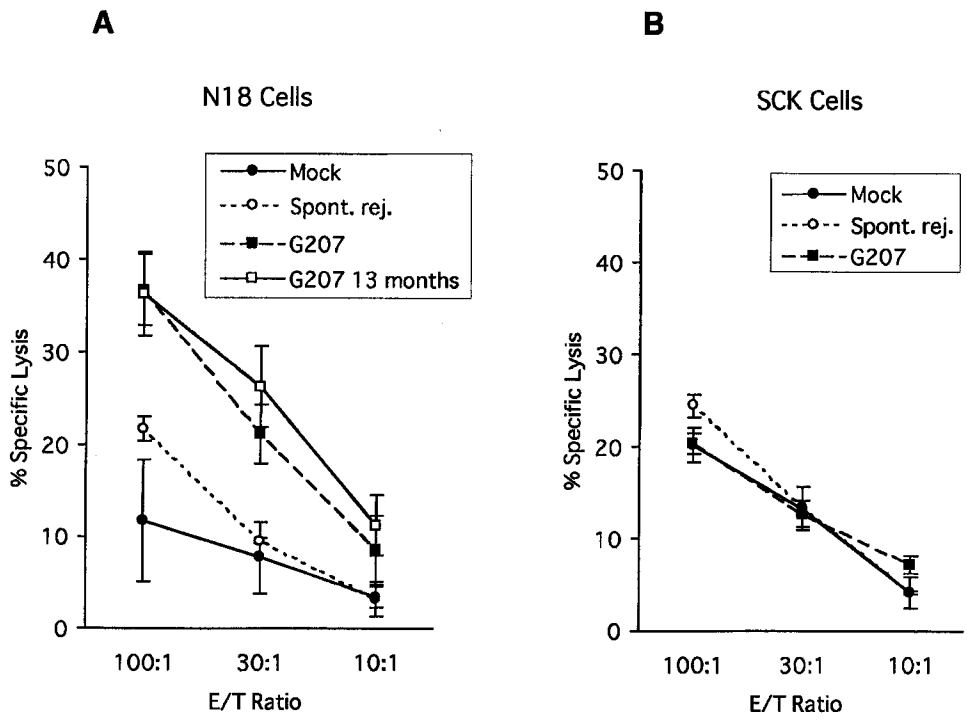


FIG. 7. G207 treatment of subcutaneous tumors induces a tumor-specific and persistent CTL activity against N18 tumor cells. Seven-week-old A/J mice harboring subcutaneous N18 tumors of approximately 6 mm in diameter were treated with an intraneoplastic inoculation of either mock-infected extract or G207 (1×10^7 PFU). The treatment was repeated on day 3. Fourteen days after the first treatment, three mice from each group (mock-treated and G207-treated groups) and three mice that received a subcutaneous injection of N18 cells but did not develop a tumor (spontaneous rejection group) were sacrificed and spleens were harvested. Splenocytes from individual animals were cocultured with irradiated N18 cells for 5 days. Effector cells of individual animals were assayed separately by a ^{51}Cr release assay, using as targets N18 cells (**A**) or SCK cells (**B**). The effector cells of the G207-treated group exhibited a significantly increased, dose-dependent CTL activity against the target N18 cells compared with those of mock-treated or spontaneous rejection groups ($p < 0.05$ at an *E:T* ratio of 100:1, unpaired *t* test). No difference in the level of lysis against the target SCK cells was observed between the three groups. Fifteen-month-old mice ($n = 3$) whose established subcutaneous N18 tumors had been cured by intraneoplastic G207 inoculations and had survived for 13 months after the subcutaneous tumor therapy showed increased CTL activity against the target N18 cells at a persisting level (**A**). The results represent the mean ± SD.

11 G207-treated mice did not develop any intracerebral tumor growth; 3 of these 11 mice were sacrificed owing to a large subcutaneous tumor burden.

To demonstrate that this antitumor effect on a remote intracerebral tumor did not derive from direct G207 infection, that is, spread of G207 from the subcutaneous tumor, PCR analysis was performed on two mice of the G207-treated group at the time of death: one mouse that died 20 days posttreatment from a brain tumor (mouse 1) and a second mouse that was sacrificed 29 days posttreatment owing to a large subcutaneous tumor with no brain tumor (mouse 2). For each mouse, the entire right frontal lobe of the brain containing the inoculation site and the entire brain tumor (mouse 1) was divided into two pieces, and DNA extracted. Because of the large size of the inoculated subcutaneous tumors (1260 and 4390 mg, respectively), four pieces (approximately 100 mg each) were cut out from different portions of the tumor and DNA was extracted. The extracted DNA was amplified for the *lacZ* portion of the G207 DNA. As expected, G207 DNA was detected in the subcutaneous tumors of both animals, with two of four specimens showing a positive band at the expected size of 300 bp (Fig. 9). In contrast, no G207 DNA was detected in the brain tissues of either mouse 1 or 2. By using mouse brain tissues spiked with various amounts of G207, the detection limit of this PCR analysis was shown to be 10 PFU (data not shown). When G207 is inoculated into a subcutaneous tumor, the virus does not readily spread throughout the entire tumor, but rather localizes to the inoculation site and the surrounding region, as demonstrated by X-Gal histochemistry (Color Plate 2A) (Mineta *et al.*, 1995; Yazaki *et al.*, 1995). Presumably, it is the noninfected tumor cells that keep proliferating and cause the continuous growth of a G207-treated tumor, which may account in part for the detection of G207 DNA in two of four specimens of each subcutaneous tumor.

In a separate experiment using the same experimental protocol, two mice of the control group and two mice of the G207-treated group were sacrificed 5 days after the first G207 inoculation into subcutaneous tumors, and the same number of animals from each group was also sacrificed 10 days postinoculation. The brain and the subcutaneous tumor of each mouse

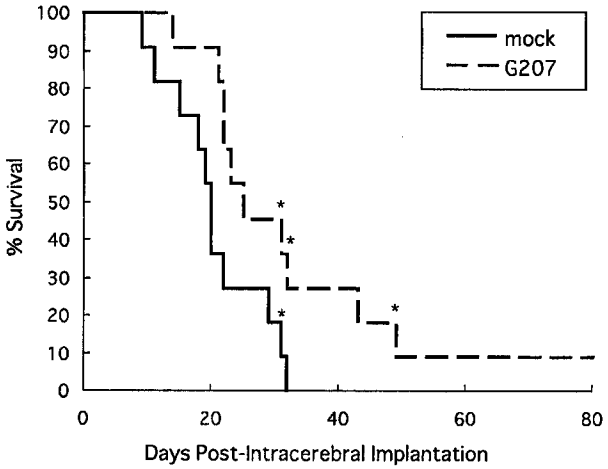


FIG. 8. G207 inoculation into subcutaneous tumors prolongs the survival of A/J mice bearing both subcutaneous and intracerebral tumors. Six-week-old A/J mice received a subcutaneous injection of 5×10^6 N18 cells in the left flank region (day 0), followed by a stereotactic intracerebral injection of 5×10^5 N18 cells into the right frontal lobe on day 3. On day 10, subcutaneous tumors were treated with an intraneoplastic inoculation of either mock-infected extract or G207 (1×10^7 PFU) ($n = 11$ per each group). The treatment was repeated on day 13. G207-treated animals showed a significant prolongation of survival compared with mock-treated animals ($p = 0.032$ by Wilcoxon test). All 11 mock-treated mice either died from or showed intracerebral tumor growth, whereas 4 of 11 G207-treated mice, including 1 long-term survivor, did not show any intracerebral tumor growth. *Sacrificed owing to a large subcutaneous tumor (≥24 mm in diameter).

were harvested, snap frozen, sectioned, and stained by X-Gal histochemistry to detect G207. In all G207-treated animals, whether from 5 or 10 days posttreatment, an abundant expression of β-galactosidase (LacZ) was observed in the virus-inoculated subcutaneous tumor, showing the presence of active G207, but, contrastingly, no histochemical staining was detected in the remote, noninoculated brain tumor (an example is shown in Color Plate 2). In all control animals, neither the subcutaneous tumor nor the brain tumor showed staining for β-galactosidase. The PCR and the X-Gal histochemistry data both indicate that G207 inoculated into the subcutaneous tumors induced an efficient antitumor effect on the remote coexisting brain tumors not by circulating and infecting the brain tumor, but most likely via systemic antitumor immunity.

DISCUSSION

Replication-competent virus vectors have been increasingly explored as a modality of viral therapy or gene therapy of cancers (Martuza *et al.*, 1991; Markert *et al.*, 1993; Jia *et al.*, 1994; Kaplitt *et al.*, 1994; Lee *et al.*, 1994; Lorence *et al.*, 1994; Mineta *et al.*, 1994, 1995; Chambers *et al.*, 1995; Kesari *et al.*, 1995; Bischoff *et al.*, 1996; Miyatake *et al.*, 1997; Pyles *et al.*, 1997; Rodriguez *et al.*, 1997; Coffey *et al.*, 1998). Although gene therapy is an attractive means of cancer treatment, none of the currently investigated nonviral or defective viral vectors has yet demonstrated 100% gene delivery *in vivo*. Hence, vector amplification within a tumor, using a replication-competent virus, is a reasonable strategy to obtain a higher efficiency. Conditionally replicating, recombinant HSV vectors with one

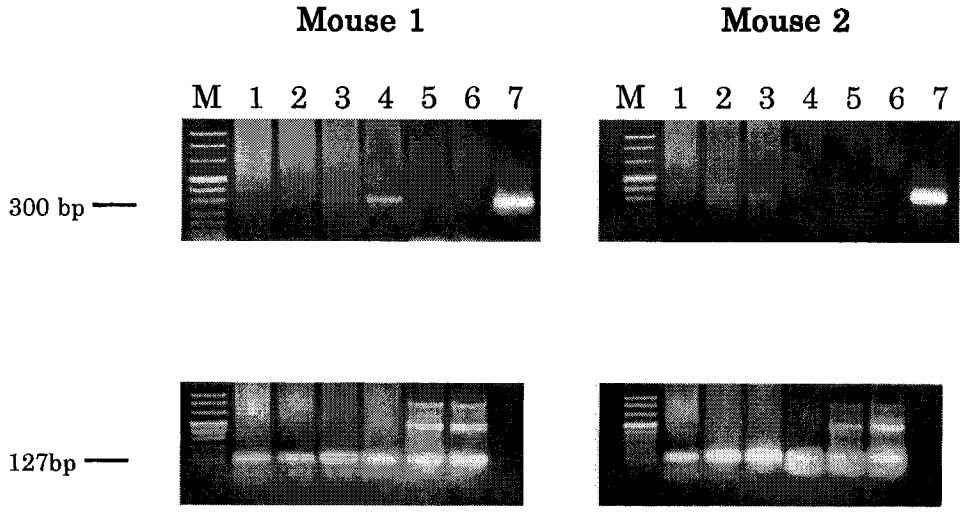


FIG. 9. G207 is not detected in the brain after inoculation into subcutaneous tumors. PCR analysis was performed on two mice bearing subcutaneous and intracerebral tumors simultaneously that received a subcutaneous tumor inoculation of G207. One mouse that died from the brain tumor (mouse 1) and a second mouse that was sacrificed owing to a large subcutaneous tumor with no brain tumor (mouse 2) were analyzed. DNA was extracted from four specimens cut from the subcutaneous tumor (lanes 1–4) and from the right frontal lobe of the brain (divided into two pieces; lanes 5 and 6). G207 DNA was used as a positive control (lane 7). A pair of primers was selected to amplify the *lacZ* portion of the G207 DNA (*top*). G207 was detected in the subcutaneous tumors (mouse 1, lanes 3 and 4; mouse 2, lanes 2 and 3), but not in the brain. Primers for fatty acid-binding protein were used as a control (*bottom*).

or more mutations in their viral genomes have been extensively investigated for viral therapy of brain tumors, and have proved to be highly efficient in killing brain tumor cells and other nonnervous system tumor cells (Martuza *et al.*, 1991; Mineta *et al.*, 1994, 1995; Chambers *et al.*, 1995; Kesari *et al.*, 1995; Yazaki *et al.*, 1995; Kramm *et al.*, 1997; Pyles *et al.*, 1997). The *in vivo* antitumor effect of conditionally replicating HSV vectors, including G207, has mostly been examined in nude or SCID mice (Chambers *et al.*, 1995; Kesari *et al.*, 1995; Mineta *et al.*, 1995; Yazaki *et al.*, 1995; Lasner *et al.*, 1996; Pyles *et al.*, 1997), or rodent models that are relatively HSV resistant (Boviatsis *et al.*, 1994; Randazzo *et al.*, 1995; Kramm *et al.*, 1996, 1997; Andreansky *et al.*, 1998). These studies have not addressed an important question as to how the host immune response would affect the antitumor therapy of a brain tumor. In an immunocompetent condition, there are several issues to be considered: (1) replication of the virus within a tumor may be inhibited by an antiviral immune response, resulting in reduction of the cytopathic effect. With adenovirus vectors, it has been shown that a strong immune response against the vector itself acts adversely to the therapeutic efficacy (Yang *et al.*, 1994, 1995); (2) on the other hand, an immune response may be associated with an antitumor immune response that provides an enhancement of the antitumor action, as observed in "suicide" gene therapy with HSV thymidine kinase gene transfer followed by ganciclovir administration (Barba *et al.*, 1994) and as has been shown with HSV outside the brain (Toda *et al.*, 1999). However, for tumors within the brain, these issues are further complicated by the immunologically privileged environment of the brain. Solving this problem is important not only for the treatment of primary brain tumors but also for the treatment of metastases to the brain.

The model we have used provides for the study of systemic and intracerebral tumors in a syngeneic host. A/J mice are the most susceptible inbred mouse strain to HSV infection. They are sensitive to most HSV strains and are killed by a low dose of HSV after intraperitoneal injection (median lethal dose [LD_{50}] of $10–10^2$) (Lopez, 1975; Zawatzky *et al.*, 1981). In comparison, BALB/c mice are susceptible to fewer HSV strains and require a 10-fold higher dose for lethality, and C57BL/6 mice are resistant to all HSV stains at the highest doses tested. While G207 is one of the most attenuated HSV vectors designed for tumor therapy and most murine cell lines are resistant to this virus, N18 cells showed a moderate susceptibility to G207. N18 cells are capable of forming a bona fide intracerebral tumor that reproducibly causes death in 2–3 weeks, and also a subcutaneous tumor with rapid growth and rare ulceration. Other favorable features of this model include an availability of cell lines cloned from the same origin, C1300, with different biological properties, e.g., Neuro2a (Amano *et al.*, 1972).

With this model, we demonstrated that G207 exhibits highly efficient antitumor action in an immunocompetent animal when inoculated intraneoplastically. One or two inoculations of G207 into an established subcutaneous N18 tumor not only caused a reduction in tumor growth but also cures in 50 to 75% of animals. The antitumor efficacy of G207 can be maximized to obtain a 100% cure rate in this tumor model by repeated intraneoplastic inoculations (Chahlavi *et al.*, 1999). We also demonstrated that this antitumor effect is accounted for, at least in part, by a systemic and specific antitumor immune response elicited by the intraneoplastic inoculation of G207. The data indicate that the induced immune response caused a reduction in the growth of an established remote subcutaneous tumor and provided long-term protection against subcutaneous rechallenge with N18 cells but not with SaI/N cells. Furthermore, the antitumor immunity was shown to be associated with the specific CTL activity against N18 tumor cells. Moreover, the elevated CTL activity proved to persist at the initial level for at least 13 months after subcutaneous tumor therapy with G207. The mechanism underlying how the replication-competent virus injected *in situ* induces a specific antitumor immune response is unclear, but the fact that virally modified tumor cells are capable of inducing antitumor immunity has been documented (Sinkovics and Horvath, 1993). This so-called postoncolytic antitumor immunity has been shown mostly by trials of immunization of the host with lysates of virally infected tumor (Lindenmann and Klein, 1967; Boone and Blackman, 1972; Wallack *et al.*, 1977; Heicappell *et al.*, 1986; Savage *et al.*, 1986). On the basis of the observation that tumor cell homogenates prepared from virus-infected tumors were highly immunogenic, whereas similar homogenates prepared by mechanical disruption and lyophilization were not immunogenic, the importance of viral replication for the adjuvanticity has been suggested (Lindenmann and Klein, 1967; Lindenmann, 1974). An HSV infection has been shown to induce production of cytokines, such as interleukin 6, from infected cells *in vitro* (Kanangat *et al.*, 1996). It has been demonstrated that cross-priming of MHC class I-restricted $CD8^+$ cells by dendritic cells occurs in the presence of antigen-specific helper T cells or viral infection (Huang *et al.*, 1994; Albert *et al.*, 1998).

We demonstrated that a single inoculation of G207 into an established N18 brain tumor results in significant prolongation of survival in syngeneic A/J mice, and that the specific antitumor immunity associated with subcutaneous tumor therapy with G207 provides a strong and persistent protection against tumor rechallenge in the brain. Furthermore, we demonstrated that established brain tumors may be inhibited in growth or even cured potentially via induction of the specific antitumor immunity elicited by G207 treatment of a remote subcutaneous tumor. Although the brain has been considered as relatively immune privileged, these results indicate that a strong antitumor immune response can occur. Regression of an established brain tumor via an *in situ* subcutaneous tumor vaccination has never been documented, but accumulating evidence suggests that activated T cells can pass through the intact blood–brain barrier to enter and function in the CNS (Hickey, 1991; Trojan *et al.*, 1993; Thompson *et al.*, 1997). It has also been documented, using mouse models, that precursors of CTLs, generated in the periphery, can traffic to tumor sites in the brain (Gordon *et al.*, 1997). Furthermore, it has been shown that actively infecting C1300 cells, the parent clone of N18, with measles virus elicits a host CTL response that lyses both infected and uninfected C1300 cells in an H-2-restricted fashion (Gopas *et al.*, 1992). These support the view that the antitumor CTL activity induced by the subcutaneous tumor therapy may have directly accounted for the remote brain tumor regression.

The present studies have several implications regarding clinical applications of G207. Immune responses after intraneoplastic inoculations of G207 were shown to contribute favorably to the antitumor effect through an elicitation of specific

antitumor immunity. The direct cytopathic effect of the replicating virus may be greatly augmented by the elicited antitumor immune response. Hence, an insufficient or uneven distribution of the virus could still lead to a cure of the tumor as observed in our mouse model. By using N18 or CT26 (murine colon cancer) tumor-bearing mice that were repeatedly exposed to HSV-1 and obtained persistent seropositivity, we have found that the presence of circulating antibodies against HSV-1 does not affect the antitumor efficacy of G207 when inoculated intraneoplastically (Chahlavi *et al.*, 1999). We have further found that corticosteroid administration does not affect *in vivo* G207 replication in N18 tumors, despite the significant suppression of serum levels of anti-G207 antibodies (Todo *et al.*, 1999). These suggest that seropositivity for HSV-1, observed in 60–90% of the adult human population (Whitley, 1996), is unlikely to limit the oncolytic activity of G207. Primary malignant brain tumors are rarely seen to metastasize outside the CNS, but metastatic brain tumors originating from a non-CNS organ are frequently encountered. Metastatic brain tumors are often refractory to systemic chemotherapy and are not always resectable owing to the location or multiplicity. The present studies introduce an immunotherapeutic approach in which treatment of the primary tumor in the periphery with G207 may lead to regression of metastatic brain tumors via induction of systemic antitumor immunity. The murine tumor model we used has many characteristics similar to human neuroblastoma (Ziegler *et al.*, 1997), a disease found mostly in children under 10 years old. Neuroblastoma occurs frequently in the abdomen, but occasionally in the CNS either as a primary or metastatic tumor, and has the highest rate of spontaneous regression of all human malignant neoplasms (Castleberry, 1997). Yet, neuroblastoma exhibits one of the poorest outcomes when occurring as disseminated disease, despite advances in postoperative treatment such as dose-intensive chemotherapy (Castleberry, 1997). Considering the potent antitumor action of G207 observed in our tumor model, neuroblastoma should also be an excellent target of viral oncolytic therapy.

It is noteworthy that none of the mice with brain tumors that were inoculated with G207 showed any morbidity or mortality from the virus itself, despite the high sensitivity of A/J mice to HSV. However, it must also be considered that, although N18 cells are derived from C1300 cell that arose spontaneously from A/J mice, their passage in culture and/or relatively high MHC class I expression among subclones of C1300 (T. Todo, unpublished data) may have been associated with increased immunogenicity. Nevertheless, N18 and its parent line, C1300, have growth and behavioral characteristics similar to human neuroblastoma: the similarities include the potential for differentiation, the potential to induce a host antitumor immune response, and a relatively high incidence of spontaneous regression (Buck *et al.*, 1977; Ziegler *et al.*, 1986, 1997; Castleberry, 1997). Despite the moderate immunogenicity of N18 tumors, the results show that the induction of a tumor rejection by the oncolytic activity of G207 leads to increased capability of the treated animals to reject remotely established or rechallenged tumors. This effect is not specific to the N18 tumor model, but has also been observed in syngeneic murine tumor models with CT26 colon cancer and Cloudman S91 clone M3 melanoma (Toda *et al.*, 1999). Still, it will be important to acquire similar data in transgenic animals or in humans. In this regard, G207 is now in clinical trial for patients with recurrent malignant glioma (Recombinant DNA Advisory Committee, 1998), and the immune effects of G207 tumor therapy are yet to be studied in patients receiving the therapy. Moreover, G207 and related vectors should be considered for treatment of systemic tumors, including those metastatic to the brain.

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